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In vitro tolerance to *Botrytis cinerea* of grapevine 41B rootstock in transgenic plants expressing the stilbene synthase *Vst*1 gene under the control of a pathogen-inducible PR 10 promoter

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Abstract

Resveratrol is a major phytoalexin in grapevine but its synthesis in response to phytopathogen attack decreases with grape berry ripening. A chimeric gene combining an alfalfa PR 10 promoter and *Vst*1 (*Vitis* stilbene synthase 1) gene was introduced into the genome of 41B rootstock. Transgenic plants were analysed for resveratrol production in leaves infected with *Botrytis* using an *in vitro* test. Among the 50 transgenic lines analysed, some exhibited a production lower than the non-transgenic control, but others accumulated resveratrol from 5–100-fold. Moreover, in the latter clones, symptoms were highly reduced in response to infection. These results were a good indication that the combination of a pathogen-inducible promoter and a defence gene may increase tolerance against fungi in grapevine. The efficacy of this approach should be further tested by experiments conducted in the vineyard.

Key words: Grapevine, *Botrytis*, stilbene synthase, inducible promoter, 'PR' proteins.

Introduction

Botrytis cinerea, which results in grey mould, and *Eutypa lata*, which is responsible for wood decay, are two fungi that cause serious diseases for grapevine, an important crop in wine-producing countries. Phytochemicals are largely used to reduce the impact of phytopathogens, but only a few compounds are effective on *Botrytis* and phytochemical treatments may also have a deleterious ecological impact on the vineyard. In addition, genetic variation of *Botrytis* under selective pressure leads to the appearance of resistant strains. Since there is no curative chemical treatment against *Eutypa lata*, infection results in plant death. Therefore, to obtain grapevine varieties resistant to these pathogens is a challenge for the future, but it is also important to keep the qualities of the must, which determine the specificity of each wine. This specificity is partly due to the use of some varieties precisely defined in the A.O.C. (Appelation d'Origine Controlée) countries. Resistant varieties might be obtained by genetic crossing with wild species, but this method is too cumbersome and long, especially for woody plants, and it may result in taste alterations. Efficient tools for the regeneration of plants via somatic embryogenesis and genetic transformation of embryogenic cells using *Agrobacterium* are available for different varieties of grape (Coutos-Thévenot *et al*., 1992*a*, *b*, Mauro *et al*., 1992, 1995*a*, *b*; Maes *et al*., 1997). It is now possible to transfer resistance genes to the genome of these varieties (Boulay *et al*., 1997). Defence mechanisms in grapevine are not well understood, but some relevant genes have been identified. The production of phytoalexins such as stilbenes is one of the major defence pathways which has been shown to

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occur in grape (Langcake and Pryce, 1977; Langcake, 1981; Pont and Pezet, 1990; Pezet and Pont, 1995; Adrian *et al.*, 1996, 1997; Coutos-Thévenot *et al.*, 1998). This production is controlled by a key enzyme, stilbene synthase, which belongs to a multigenic family (Wiese *et al.*, 1994). Transgenic tobacco plants overexpressing a stilbene synthase gene from grape show resistance against *Botrytis* (Hain *et al.*, 1993; Fischer and Hain, 1994). Stilbene synthase condenses three malonyl CoA molecules with one molecule of Coumaryl CoA to produce resveratrol. This diphenol compound is metabolized, producing ε-viniferin (dimerization), pterostilbene (methylation) and piceid, a resveratrol glucoside (Fig. 1). These compounds represent the major forms of phytoalexins in grape. Resveratrol accumulation dramatically decreases 16 weeks post-flowering (Bais *et al.*, 2000), and is very low just before harvest. This decrease may be due to a limitation in substrate availability, which may result either from decreased synthesis of coumaryl CoA and malonyl CoA or from competition between chalcone synthase and stilbene synthase using the same substrates (Fischer *et al.*, 1997) (Fig. 1).

In the work described, the coding sequence of *Vst*1 from grapevine (Wiese *et al.*, 1994) has been fused to

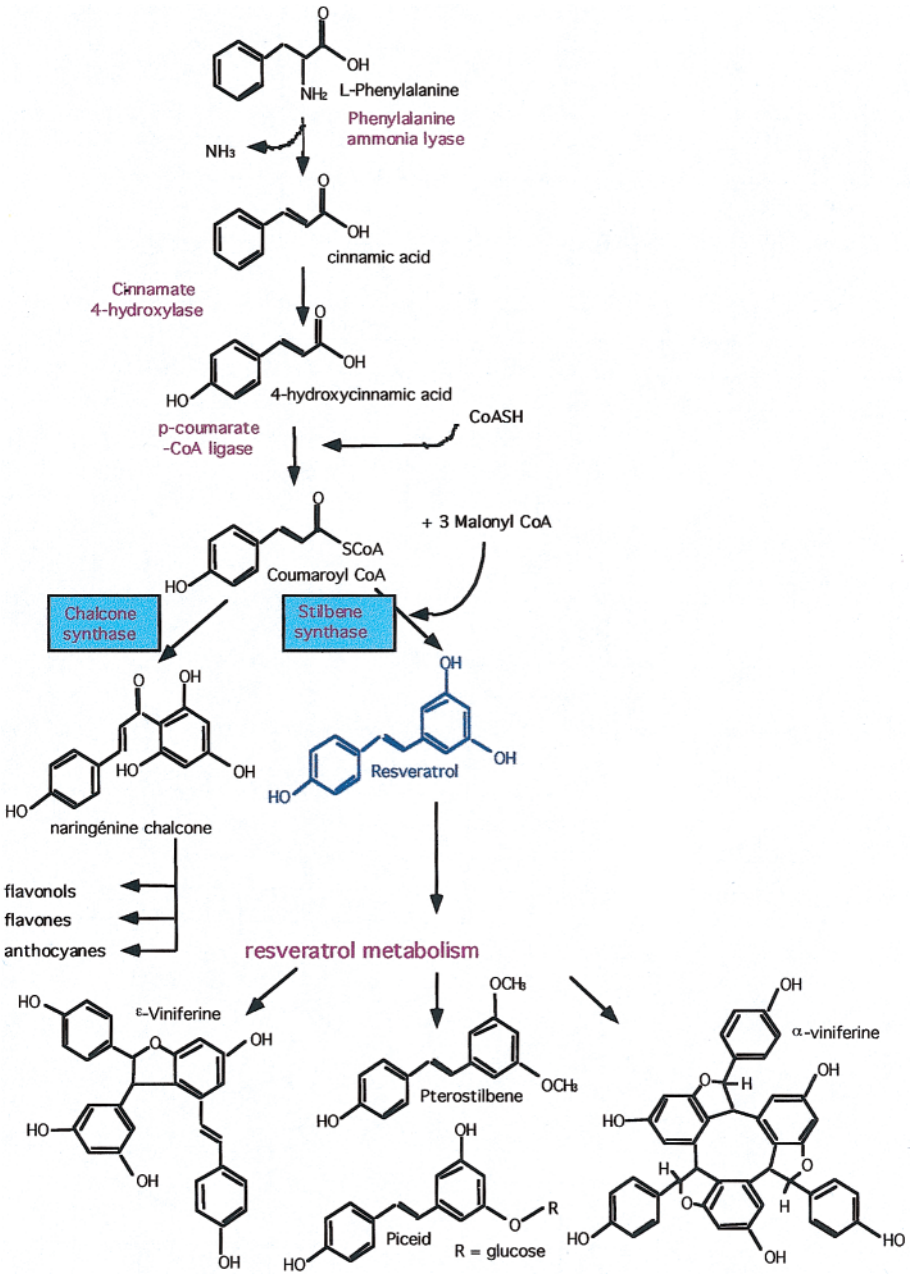

Fig. 1. Stilbene and chalcone biosynthesis pathways in grape and major metabolites derived from resveratrol.

a fungal-inducible promoter (Coutos-Thevenot *et al*., 1998; Esnault *et al*., 1998). The promoter used belongs to a class 10 PR (pathogenesis related, accession number X98867) gene expressed in alfalfa upon incompatible interaction with *Pseudomonas syringae* pv. *pisi* (Esnault *et al*., 1993; Breda *et al*., 1996). After genetic transformation via *Agrobacterium* harbouring this construct in a binary vector, an *in vitro* test of infection with *Botrytis* on transgenic microcuttings has been developed to monitor resveratrol accumulation and the extent of symptoms.

Materials and methods

Fungi

The *Botrytis cinerea* strain 916T (B Dubos, INRA, Bordeaux) and the *Eutypa lata* BK 123 8 d strain (JP Peros, ENSAM-INRA, Montpellier) were multiplied in 9.5 cm Petri dishes with 20 ml half-strength V8 culture medium (commercial vegetable juice V8) supplemented with 5 g l^{-1} KH_2PO_4, 30 g l^{-1} bacto agar (pH 6.0) and subcultured each month. Conidia production in *Botrytis* was obtained by exposing Petri dishes to the light.

Fungus growth inhibition

A fungal inoculum was placed in the centre of a Petri dish containing a malt-agar culture medium (15 g l^{-1} malt extract, 20 g l^{-1} bacto agar, pH 6.0) supplemented with different concentrations of resveratrol, solubilized in ethanol (1% final concentration in the malt-agar culture medium). The control was supplemented with the same quantity of ethanol. Fungal growth was expressed as the diameter of the mycelium spreading from the inoculum point. Growth inhibition was calculated by comparison with the control without resveratrol treatment.

Grape transformation and culture conditions

The 41B rootstock (*Vitis vinifera* cv. Chasselas × *Vitis berlandieri*) was chosen as an experimental model. An embryogenic cell suspension culture was initiated as described previously (Mauro *et al*., 1992, 1995*a*). This cell suspension was subcultured each week in 25 ml of modified MS liquid culture medium (Coutos-Thevenot *et al*., 1992*a*) supplemented with synthetic auxin (naphthoxy acetic acid, NOA, 1 mg l^{-1}) in the dark. Embryogenic cells were transformed using an *Agrobacterium* cocultivation method (Mauro *et al*., 1995*a*), and after selection, transgenic plantlets were regenerated by somatic embryogenesis (Coutos-Thevenot *et al*., 1992*a*) and propagated as *in vitro* microcuttings. Each transgenic line derived from one embryo represents a putative clone. These different lines were multiplied separately (50 different clones per transformation experiment).

For all the experiments described below, non-transgenic plants and the primary transformants were multiplied by internode microcuttings in MS culture medium (Murashige and Skoog, 1962) supplemented with 7.5 g l^{-1} agar, pH 5.8 in MAGENTA™ boxes (Sigma). The microcuttings were maintained for 45 d at 24 °C during the day and 18 °C at night, 16 h light at 250 μE m^{-2}, and the plants obtained in this way were used for experiments.

Biotic and abiotic stresses

Infiltration of *Nicotiana benthamiana* with *P. syringae* pv. *pisi* was as described previously (Esnault *et al*., 1993). Grape infection by *Botrytis* or irradiation by UV light were performed on four different leaves (same rank), coming from four different 45-d-old plants. For *Botrytis* infection, 25 μl of conidia suspension (10^4 conidia ml^{-1}) in 15 g l^{-1} malt extract and 0.1 M glucose medium were inoculated at the surface of the leaves using a micropipet and plants were cultivated *in vitro* in MAGENTA™ boxes (Sigma). After 9 d of incubation, the inoculated leaves were excised, frozen in liquid nitrogen and stored at −80 °C. Abiotic stress was achieved by irradiating the entire plant with UV light at 254 nm for 8 min with an energy of 270 μW m^{-2}. Samples were collected 17 h after irradiation as described above.

Resveratrol quantification

Frozen leaves were ground with a mortar and pestle in liquid nitrogen and resveratrol was extracted with 1 ml of methanol per 100 mg FW. The methanolic suspension was transferred to Eppendorf tubes and centrifuged 10 min at 13 000 *g*. To remove chlorophylls, the supernatant was passed through a Sep-Pack® C-18 cartridge (Waters, USA) equilibrated with methanol. The filtrate was evaporated under nitrogen flux and the extract was solubilized in 1 ml of methanol and clarified by filtration through Millex FGS 0.22 μm (Millipore) before HPLC analysis.

Resveratrol was quantified with an HPLC system (Merck L-5200) coupled to an automatic injector (Merk AS-4000 PL). Samples (25 μl of each) were loaded onto a silica C-18 reverse phase column (Kromasil C-18, 250 × 3 mm, 5 μm) equilibrated with an acetonitrile/H_2O (5/95 v/v) mobile phase (solvent A) at a flow rate of 0.8 ml min^{-1}. The bound molecules were eluted with a step gradient of solvent B (acetonitrile/H_2O, 95/5 v/v). The gradient was formed according to the following steps: 0–10 min, 35% of B; 10–20 min, 50% of B; 20–30 min, 80% of B. Elution was monitored by OD at = 305 nm, the optimal absorption of resveratrol. Quantification was obtained after injection of different quantities of 3,4′5-trihydroxy-*trans*-stilbene (99% purity), the natural resveratrol (Sigma) to establish the calibration curve as a function of integrated peak area. Results were expressed in μg of resveratrol g^{-1} DW. To identify the peak corresponding to resveratrol, the UV absorption spectra between 200 and 400 nm (diode array (Waters, USA) coupled to the HPLC) of each eluted peak was compared with the spectrum obtained from the commercial standard.

RNA blots

Total RNAs were extracted from 1 g of leaves according to the method described earlier (Tesnière and Vayda, 1991). Leaves were ground in liquid nitrogen to a fine powder and homogenized in extraction buffer (0.2 M TRIS-HCl, 1.5% SDS (w/v), 0.3 M LiCl, 0.01 M EDTA, 1% sodium deoxycholate, 1 mM aurintricarboxylic acid, 5 mM thiourea, and 1% Nonidet P-40 (v/v)). After centrifugation, the supernatant was purified three times using phenol/chloroform/isoamyl alcohol (25/24/1 by vol) extractions and the nucleic acids were ethanol precipitated. The RNAs were then purified by a LiCl (2 M final) differential precipitation and quantified by OD at 260 nm.

The *Vst*1 stilbene synthase coding sequence (accession number S63225) was excised from Vst1 plasmid (gift of Bayer, Germany) and subcloned in the pCDNA II plasmid (*InVitrogen*, USA) as an EcoRI/PstI fragment (pCDNA II-Vst1). RNA antisense DIG (digoxygenin) labelled probe was produced by

in vitro transcription (DIG RNA labelling kit, Boehringer) using T7 RNA polymerase after EcoRI plasmid linearization.

RNAs were loaded (25 µl per lane) onto a denaturating 1.2% agarose gel, containing MOPS 1×, 38.5% (v/v) formamide and 2.2 M formaldehyde (Maniatis *et al*., 1982). After migration, the RNAs were blotted onto Hybond N^+ (Amersham) nylon membrane, cross-linked by exposure to UV light at 254 nm and methylene blue stained (Maniatis *et al*., 1982). This was used to determine if equal amounts of RNAs were loaded in each lane. The membrane was hybridized (DIG easy Hyb buffer, Boehringer) at 50 °C overnight, washed twice in 0.5×SSC, 0.1% SDS at room temperature and once in 0.1×SSC, 0.1% SDS at 50 °C. The hybridization was revealed by a chemiluminescent technique using the DIG detection kit (Boehringer) and the CDPStar chemiluminescent substrate (Boehringer).

Gus activity

Twenty-four hours after induction, leaves were treated with 2 ml of GUS solution (50 mM NaH_2PO_4, 1 mM X-gluc, 0.5 mM $K_3Fe(CN)_6$, and 0.5 mM $K_4Fe(CN)_6$ (Jefferson *et al*., 1987) and incubated overnight at 37 °C. Then samples were fixed in 10 ml of 70% ethanol solution (v/v).

Molecular techniques

A genomic library of alfalfa was screened using a cDNA probe of a class 10 PR protein (MsPR10.1, accession number X98867, Breda *et al*., 1996) and a 1.5 kb promoter fragment (Pr10prom) was cloned in pBKS$^+$ plasmid as described previously (Esnault *et al*., 1998). PR 10 promoter was subcloned as an EcoR1/Kpn1 fragment in the pBIN 19 binary vector, then the Vst1 coding sequences was inserted downstream of this promoter as described previously (Coutos-Thevenot *et al*., 1998). The construct (pBIN-19-Pr10prom-Vst1) was transferred into LBA 4404 *Agrobacterium* strain by triparental conjugation. The T0 *Nicotiana benthamiana* plants transformed by using the transgene Pr10prom/*uid*A, were provided by Dr P Ratet (Institut Sciences Végétale, CNRS). Southern analysis indicated that five out of eight regenerated plants showed the transgene integration. Analysis of the GUS activity were conducted on T1 plants.

Results

Inhibition of mycelium growth by resveratrol

The inhibitory effect of resveratrol on *Botrytis* mycelium growth has been a matter of debate (Pont and Pezet, 1990; Adrian *et al*., 1997). In this context, before developing the genetic transformation of grape, several concentrations of commercial trans-resveratrol (Sigma) were tested to check the validity of this strategy. The two grape phytopathogens chosen, *Botrytis* and *Eutypa*, were selected because of their economical importance. Fungal growth was expressed as the diameter of the mycelium spreading from the inoculum point as described in Materials and methods. For both fungi, a significant and increasing growth inhibition was observed for concentrations ranging between 75 and 500 µM resveratrol (Fig. 2). At the highest concentrations tested (above 500 µM), inhibition was higher for *Botrytis* than for *Eutypa*, but in no case

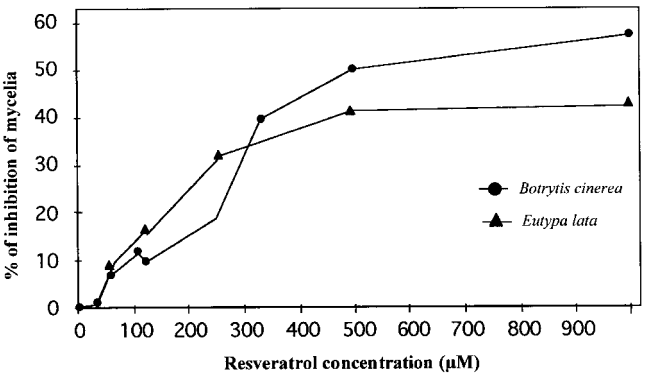

Fig. 2. Growth inhibition of *Botrytis cinerea* and *Eutypa lata* mycelia as a function of resveratrol concentration. Resveratrol was solubilized in 1% ethanol (final concentration) in malt-agar culture medium. The control was supplemented with the same quantity of ethanol. The percentage of inhibition was calculated as: % of inhibition = (growth diameter of the control)−(growth diameter of the treated)/(growth diameter of the control)×100. Growth diameter used was the average of two perpendicular diameter measurements of the mycelium growth. Data are means of two independent experiments.

was total inhibition observed. This may be due to the low solubility of resveratrol, which results in its partial precipitation above 500 µM, even in 2% ethanol.

The effects of resveratrol on conidia germination were tested in the same conditions (solid medium). All the conidia inoculated on 500 µM resveratrol were able to germinate, but after germination, mycelium growth was strongly decreased compared to the control (data not shown).

Production of resveratrol and stilbene synthase transcript accumulation under biotic and abiotic stress conditions

Expression of the stilbene synthase gene under the control of its own promoter is inducible by a biotic stress like *Botrytis* infection and also by an abiotic stress (UV light at 254 nm). Before investigations on transgenic grapevine, resveratrol production in the *in vitro* 41B rootstock and in different varieties of grapevine was measured to quantify the natural phytoalexin production in the plant system investigated (Table 1).

Non-induced *in vitro* plants do not produce detectable levels of resveratrol. Under these conditions, no stilbene synthase transcripts could be detected by RNA blots analysis (Fig. 3, lane 1).

It was estimated that after induction by *Botrytis*, resveratrol concentrations in leaves of non-transgenic plants cultivated *in vitro* in MAGENTA™ boxes were in the range of 40–140 µg g^{-1} DW. The varieties tested did not differ significantly in their *Botrytis*-induced resveratrol production. After 20 d of culture, mycelium of *Botrytis* was fully developed and induced plant death under the *in vitro* conditions used (high humidity and presence of sucrose in the plant culture medium).

Table 1. *Effects of* Botrytis *infection or UV treatment on resveratrol accumulation by various wild-type grapevine cultivars*

Data are means of four independent experiments ± SE (standard error).

Variety	Control not induced	*Botrytis* Resveratrol (μg g^{-1} DW)	UV light Resveratrol (μg g^{-1} DW)
Rupestris	nd	nd	350 ± 115
41B Rootstock	nd	112 ± 30	240 ± 120
Ugni blanc 479	nd	86 ± 45	210 ± 74
Pinot noir 386	nd	103 ± 31	87 ± 49
Folle blanche	nd	101 ± 16	38 ± 11

Resveratrol was quantified by HPLC (see Materials and methods) and expressed as μg g^{-1} DW on 45-d-old plants cultivated *in vitro* as described in Materials and methods. Extractions were performed on four leaves (same rank) coming from four different plants of identical age 2 d after inoculation with *Botrytis* or 17 h after UV treatment. nd: not detected.

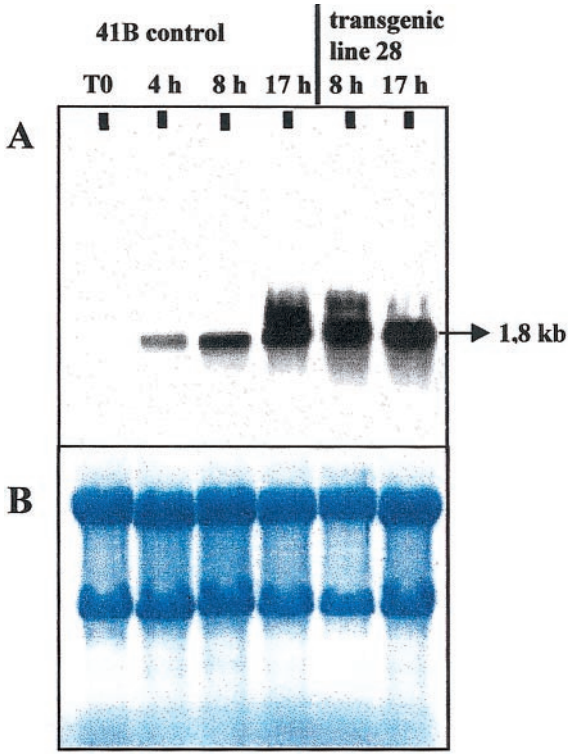

Fig. 3. RNA blot analysis of stilbene synthase transcripts in control and UV treated 41B plants. Non-transformed 41B micropropagated *in vitro* as described in the Material and methods were used as control. The transgenic line 28 has incorporated the construct pBIN 19-Pr10 prom-Vst1 chimeric gene. 25 μg of total RNA were loaded in each lane. (A) Hybridization with the complete cDNA *Vst*1 fragment as a probe. (B) Methylene blue staining.

For UV treatments, 45-d-old plants micropropagated in MAGENTA™ boxes were induced and analysed as described in Materials and methods. All the varieties tested also accumulated resveratrol in response to UV light, but the level of accumulation depended on the variety (Table 1), with Rupestris 215 being the strongest accumulator (350 ± 115 μg g^{-1} DW), and Folle Blanche the lowest one (40 ± 11 μg g^{-1} DW). Variability between different plants of the same variety was high, which also was observed previously for UV stress (Sbaghi, 1993). In the 41B rootstock, the levels of stilbene synthase transcripts were significantly increased as early as 4 h after UV treatment, and a much stronger accumulation was observed at 17 h (Fig. 3).

Alfalfa PR 10 promoter activity in tobacco

In alfalfa (*Medicago sativa*), *Pseudomonas syringae* pv. *pisi* induces a hypersensitive response (HR) due to an incompatible reaction involving the production of several PR proteins (Esnault *et al.*, 1993). A gene family corresponding to a class 10 PR protein is strongly expressed around the necrotic zones (Breda *et al.*, 1996). An alfalfa genomic fragment of 6.1 kb containing the Ms PR10.1 gene sequence has been cloned. A chimeric construct in which the MS PR10.1 promoter (1.5 kb DNA fragment) is fused with a *uid*A gene has been used to transform *Nicotiana benthamiana*plants in order to study promoter activity during interaction with pathogens. Infiltration of these transgenic *Nicotiana* leaves with *Pseudomonas syringae* pv. *pisi* induced a high expression of the *uid*A gene in mesophyll cells and in the veins (Fig. 4B) compared to control leaves infiltrated with 10 mM $MgCl_2$ (Fig. 4A). These results indicated that the alfalfa promoter was highly induced during plant–pathogen interaction and might be used to regulate expression of some resistance genes in a genetic transformation programme.

Expression of the chimeric gene Pr10prom-Vst1 in transgenic 41B rootstock grapes and accumulation of resveratrol in leaves

After genetic transformation of 41B embryogenic cells and regeneration of transgenic grape plants according to the method previously described (Coutos-Thevenot *et al.*, 1992*a*, *b*; Mauro *et al.*, 1992, 1995*a*, *b*), 50 independent transgenic lines were obtained. The primary transformants were micropropagated as described in Materials and methods. Eleven stilbene synthase genes are present in the grapevine genome as well as several chalcone synthase genes which are 70% identical to stilbene synthase. This would make it difficult to interpret Southern blots obtained with the vst1 probe. Thus, to determine if these plants were really transformed, a Southern blot analysis was performed using the *nptII* (neomycin phosphotransferase) gene as a probe (data not shown). Although the use of an *nptII* probe is an indirect approach to test transformation by the chimeric gene, this approach was validated by measurement of resveratrol accumulation (Fig.5). Expression of the Pr10prom-Vst1 chimeric gene under different stress conditions was studied. RNA blot analysis revealed that stilbene synthase transcripts were highly induced by UV stress in the

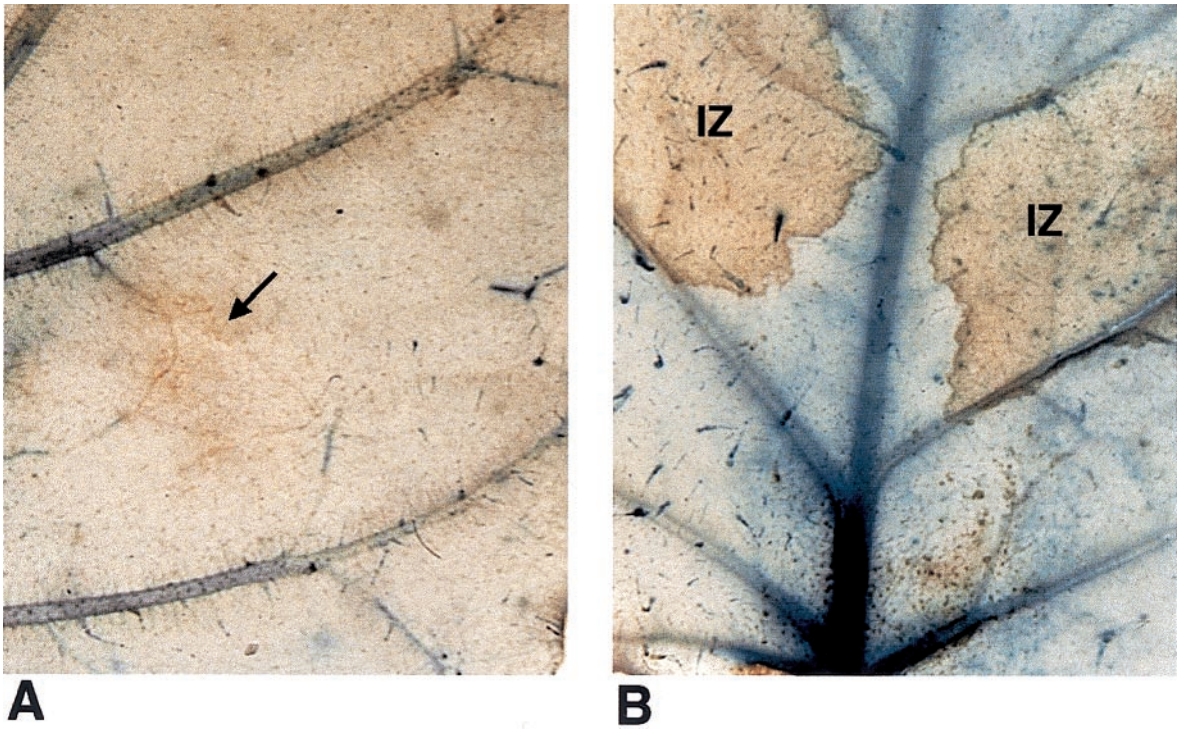

Fig. 4. Expression of the *uid*A gene under the control of the PR 10 promoter of alfalfa in transgenic *Nicotiana benthamiana* leaves after infiltration with a *Pseudomonas syringae*. Visualization of the GUS activity was performed as described in the Material and methods. (A) Control leaf infiltrated (arrow) with $MgCl_2$ 10 mM. (B) Leaf of plant infiltrated with the bacteria. IZ: infiltrated zone.

transgenic line 28 (Fig. 3, right lanes) as soon as 8 h after the beginning of treatment, and continued to accumulate at 17 h. RNA analysis on stilbene synthase after *Botrytis* inoculation was not possible due to the strong RNA degradation observed in infected control plants. Therefore, resveratrol accumulation was measured 4 d after infection, directly in the leaves of control and 15 putative independent transgenic lines infected with the fungus (Fig. 5).

There were three distinct groups of transgenic lines. The first group (for example, line 12) accumulated resveratrol at the same level (20 μg g^{-1} DW) or less than the control. In another group (i.e. lines 2, 18, 21), resveratrol accumulation was 2–10-fold higher than in the control. This difference was significant and found in three independent series of experiments. In the third group (line 28), resveratrol accumulated at a very high concentration (2000 μg g^{-1}), about 100-fold over the control 4 d after the beginning of infection.

Resveratrol, a diphenol molecule, fluoresces blue when it is excited at 365 nm. This allows its visualization in leaf tissues by epifluorescence microscopy. Visualization of resveratrol in the leaves of the transgenic line 28 showed a more intense blue fluorescence in the mesophyll cells around the location of inoculation, and also in the veins (Fig. 6B) when compared to the control (Fig. 6A).

The macroscopic symptoms for lines 28, 5 and 12 (this latter line identical to the control in terms of resveratrol production) was observed during 21 d of culture (Fig. 7). Three plants of each line were tested and the experiment was repeated three times independently. The control

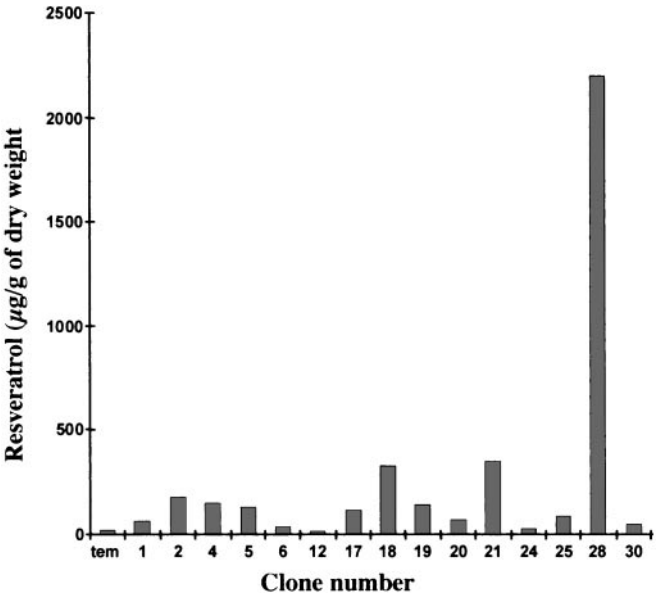

Fig. 5. *Botrytis*induction of resveratrol accumulation in different grape lines transformed with the Pr10 promoter-stilbene synthase construct. The plants prepared *in vitro* as described in the Materials and methods were inoculated with 200 conidia on the three youngest leaves. After 4 d of incubation, leaves were sampled, resveratrol extracted and quantified by HPLC.

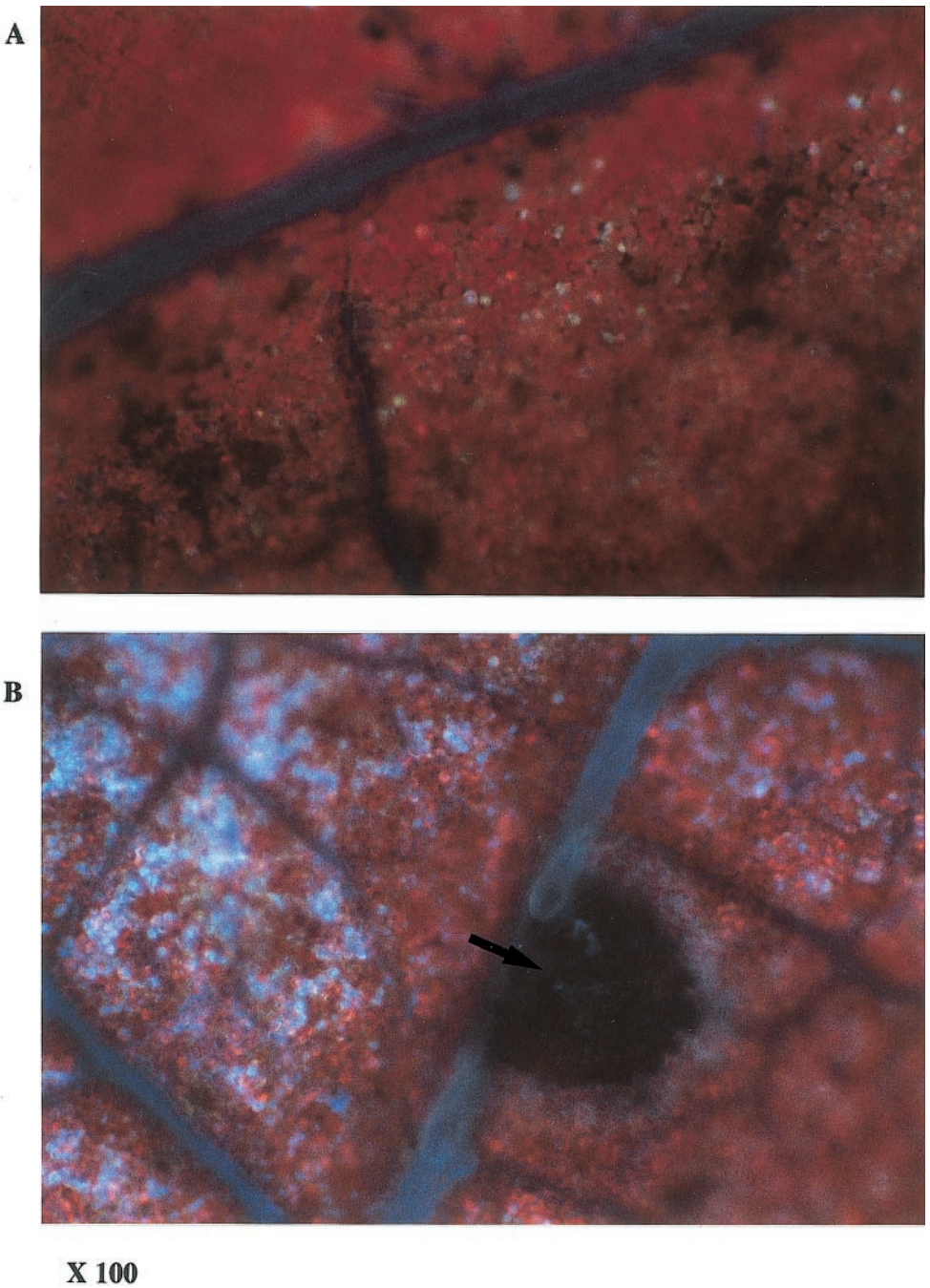

Fig. 6. Visualization of resveratrol in leaf mesophyll of 41B plants after infection with *Botrytis cinerea* conidia. Four days after inoculation, leaves were observed with a fluorescence microscope (excitation was fixed at 365 nm and emission was higher than 440 nm). Blue colour corresponded to phenol molecules and red colour to chlorophylls. Arrow indicates conidia infection site. (A) 41B non-transgenic plants (control). (B) Transgenic line 28.

plant (Fig. 7A) exhibited very severe symptoms of disease and the mycelium contaminated the surface of the plant culture medium. For transgenic line 28, the presence of mycelium was not detectable macroscopically (Fig. 7B) and plant growth was not affected compared to a non-contaminated control. On the leaves of these transgenic plants, necrotic spots were only seen at the site of infection. All these results indicated that line 28 was tolerant of *Botrytis* infection under *in vitro* conditions. Moreover, the same test conducted with line 5 which accumulated only 5-fold resveratrol compared to the control revealed a reduction of the symptoms (Fig. 7C). In many cases, mycelium was visible on leaves at the end of the experiment but plant growth was not affected.

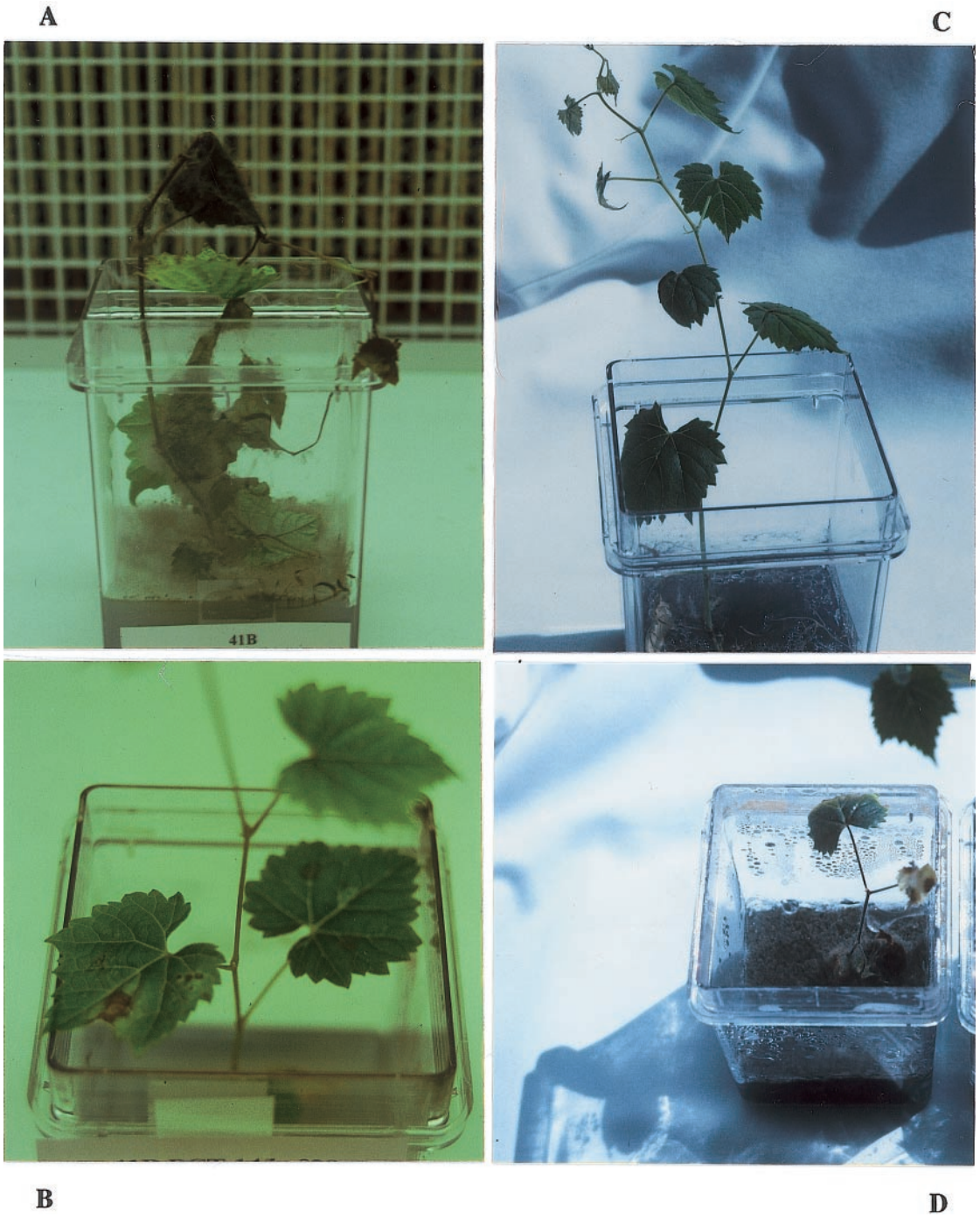

Fig. 7. Macroscopic symptoms of *in vitro* 41B plants 20 d after interaction with *Botrytis cinerea*. Plants were infected with 200 conidia in Magenta® box. (A) 41B untransformed plant as control. (B) Transgenic 41B line 28. (C) Transgenic 41B line 5. (D) Transgenic 41B line 12.

For the transgenic line 12 (Fig. 7D) that has a resveratrol level lower than the control, all the nine plants tested were completely infected after 10 d of culture.

To have statistical confirmation that transgenic line 28 shows an increased resistance to *Botrytis*, 15 plants of both non-transgenic and transgenic line 28 were inoculated with conidia in the same conditions as above. The macroscopic evolution of symptoms was observed for 3 weeks. Susceptibility to grey mould infection was evaluated by the appearance of mycelium on infected leaves. After 1 week, differences between control and clone 28 were detectable (Table 2). At the end of the test, all inoculated leaves of control (45/45) were contaminated while for transgenic clone 28 the growth of mycelium was very low and not detected on more than 40% of inoculated leaves. For these leaves, the formation of

Table 2. *Susceptibility of transgenic line 28 to* Botrytis *cinerea infection*

	Total number of leaves tested	Number of leaves showing *Botrytis* mycelium growth at different times after inoculation				
		4 d	7 d	10 d	15 d	21 d
Control	45	0	26	35	38	45
Clone 28	45	0	12	24	24	26

Fifteen plants were prepared in Magenta boxes and inoculated with *Botrytis* conidia as described in Materials and methods.

black necrotic spots was also detected at the infection site. These results confirmed, on a higher number of plants, that clone 28 exhibits tolerance against grey mould under *in vitro* conditions.

Discussion

The results reported here indicate that genetic transformation of grapevine with the chimeric Pr10prom-Vst1 construct increased resveratrol production during fungal infection, due to the expression of the stilbene synthase gene under the control of this pathogen-inducible promoter. Among the 30 transformants analysed by HPLC, eight plants showed an over-accumulation of resveratrol. In addition, plants grown *in vitro* that overproduce resveratrol are more tolerant to *Botrytis*. The idea underlying the use of an inducible promoter was to express the gene only in response to pathogen attack. Moreover, resveratrol is already present in wine (10 mg l^{-1} in red wine) and has been cited to act against cardiovascular heart diseases for moderate consumers (Frankel *et al*., 1993; Carbonneau *et al*., 1997).

The decrease of *Botrytis* infection for the transgenic lines 28 and 5 (Fig. 7; Table 2) seems to be related to a higher level of resveratrol in the transgenic plants (Fig. 5) because line 12, with a very low level of resveratrol in leaves, was sensitive. In addition, resveratrol applied directly to the fungus inhibited *Botrytis* and *Eutypa* growth *in vitro* (Fig. 2). Growth inhibition of the fungi *in vitro* was observed at high resveratrol concentrations (100 μM and higher). In the transgenic line 28, for example, resveratrol was estimated at 2000 μg g^{-1} DW (Fig. 5). Assuming that water represents 90–95% of the leaf tissue (*in vitro*), the resveratrol level in fresh leaves of line 28 was aproximately 200 μg g^{-1} FW. Although this is a crude estimation, resveratrol concentration in line 5 would be around 0.1 mM. The estimate of the *in vivo* concentration of resveratrol in line 28 falls in the range of the *in vitro* concentration inhibiting mycelium growth observed in Fig. 2.

A high resveratrol level in some particular varieties of grape (*V. rupestris* for example) has been shown to confer a tolerance to powdery mildew (*Uncinula necator*) and downy mildew (*Plasmopora viticola*) (Daï, 1994). Whether the transgenic grapes prepared in the present work also exhibit some tolerance to these other diseases should be checked in the future.

Data presented here were obtained *in vitro* and it will be important to determine if the transgenic grapevines grown in the vineyard, and more particularly berries, are tolerant. Indeed, whether this promoter is induced in the berries, the main site of *Botrytis* infection, is unknown. Unfortunately, it is necessary to wait 3 or 4 years for the first flowering period after planting. These experiments are under way.

It is also important to determine the effect of high resveratrol accumulation on both the physiology of the grape in the field and the qualities of the must. Constitutive expression of stilbene synthase under the control of the 35S promoter does not alter the normal growth of tobacco plants (Hain *et al*., 1993) but it could alter that of grapevine plants. Accumulation of resveratrol might modify the colour of the flowers, alter pollen maturation leading to male sterility, and morphologically alter flowers (Fisher *et al*., 1997). These effects, probably due to a competition between stilbene synthase and chalcone synthase for coumaroyl CoA, may limit the use of stilbene synthase in transformation experiments. Yet, the pathogen-inducible character of the PR10 promoter could minimize these effects in our plants, since stilbene synthase expression and resveratrol synthesis are limited to the time and location of infection. Finally, it will be important to determine if resveratrol alters the fermentation processes of the must into wine.

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